PREPARATION OF CATION-EXCHANGE MEMBRANE CHROMATOGRAPHY BY MODIFICATION OF POLYAMIDE MEMBRANE THROUGH GRAFTING OF METHACRYLIC ACID MONOMER

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ABSTRACT

Nowadays, with the increase demand of protein production and purity, the purification cost of the protein also increases about 50 to 90%. Packed bed chromatography is widely used in protein separation. However, there are some limitations of the packed bed chromatography such as high-pressure drop, channeling problem and complicated scale up process. Most of these limitations can be overcome by membrane chromatography. The main objective of this research is to prepare cation-exchange membrane chromatography from polyamide membrane by chemical grafting of methacrylic acid monomer. Potassium persulfate and potassium metabisulfite was used to generate the radicals in the polyamide membrane and grafted with methacrylic acid monomer together with ethylene glycol dimethacrylate as a cross-linker. Different monomer concentration between 0.1 and 1.0M and monomer grafting duration from 15 – 120 min was studied toward producing high protein binding capacity membrane. Optimum time for grafting methacrylic acid onto polyamide membrane was 45 minutes with the binding capacity 599.6390 mg/g BSA at the monomer concentration of 0.1 M. Identification and optimization of critical parameter in grafting process is crucial for the development of high performance membrane chromatography materials.

ABSTRAK

Pada masa kini, dengan meningkatnya permintaan pengeluaran protein dan juga ketulenan protein, kos pemisahan protein juga meningkat kira-kira 50 hingga 90%. Kromatografi turus terpadat digunakan secara meluas dalam proses pengasingan protein. Walau bagaimanapun, terdapat beberapa kekangan bagi kromatografi turus terpadat seperti penurunan tekanan yang tinggi, masalah penyaluran aliran dan juga proses pengembangan skala yang rumit. Kebanyakan kekangan ini dapat diatasi dengan penggunaan membran kromatografi yang mempunyai kejatuhan tekanan yang rendah, proses pengembangan skal yang mudah dan boleh beroperasi pada kadar pemprosesan yang tinggi. Objektif utama kajian ini adalah untuk menyediakan membran kromatografi jenis penukar cas positif dari membran poliamide menggunakan asid metakrilik sebagai monomer. Kalium persulfate ($K_2S_2O_8$) dan kalium metabisulfite ($K_2S_2O_5$) digunakan untuk menjana radikal dalam membran poliamide dan ditambah dengan monomer asid metakrilik bersama-sama dengan dimetilkrilik glikol etilena sebagai agen pemaut. Kepekatan monomer yang berbeza iaitu diantara 0.1 dan 1.0M dan tempoh cantuman monomer dari 15 - 120 minit telah dikaji kearah menghasilkan pengikatan protein yang berkapasiti tinggi. Keputusannya, masa optimum bagi poliamide yang telah diubahsuai permukaanya adalah 45 minit dengan kapasiti penjerapan sebanyak 599.6390 mg/g BSA pada kepekatan monomer 0.1 M. Penentuan dan pengoptimuman parameter penting semasa proses *grafting* adalah mustahak dalam menghasilkan membrane kromatografi berkapasiti terbaik.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA	Acrylic Acid
ATR-FTIR	Attenuated Total Reflection Fourier Transform Infrared
BSA	Bovine Serum Albumin
C_o	Initial Concentration
C_f	Final Concentration
Ca	Calcium
cm	Centimeter
DI	Deionized Water
EGDMA	Ethylene Glycol Dimethacrylate
ELISA	Enzyme-Linked Immunosorbent Assay
HCl	Hydrochloric Acid
K	Potassium
kPa	kiloPascal
$K_2S_2O_5$	Potassium Metabisulfite
$K_2S_2O_8$	Potassium Persulfate
$LiNO_3$	Lithium Nitrate
m	Mass of Substance
M	Molarity
MA	Methacrylic Acid
meq	miliEquivalent
mg	Milligram
ml	Milliliter
M_w	Molecular Weight
Na	Sodium
NaOH	Sodium Hydroxide
nm	Nanometer
NMP	N-Methylatedpyrrolidone
PA	Polyamide
PEG	Polyethylene Glycol
PEGMA	Polyethylene Glycol Methacrylate
PI	Isoelectric Point
pKa	Acid Dissociation Constant
Q	Binding Capacity
Redox	Reduction and Oxidation
RO	Reverse Osmosis
rpm	Rotation Per Minute
SPM	Sulfo-Propyl Metacrylate
SPS	Sulfonated Polysufone
TFC	Thin-Film Composite
UF	Ultrafiltration
UV	Ultra Violet
V	Volume
W	Weight of Dry Membrane

W_C	Water Content
W_W	Weight of Wet Membrane

CHAPTER 1

INTRODUCTION

1.1 Background

Large scale separation and purification step may contribute a large extend of the total product manufacturing cost. Sometimes, the cost of protein purification and protein separation can be as high as 50 to 90% of the total production cost (Saiful et al, 2006).

Several techniques are available in protein separation such as chromatography, membrane filtration, centrifugation, precipitation and membrane chromatography. Among them, chromatography based separation is widely used and more specifically using packed bed chromatography (Saufi, 2010).

Chromatography is the separation process in which a mixture of components to be separate is carried by a mobile phase (i.e. solvent) to pass through a stationary phase (adsorbent) (Berezkin et al, 2006). Each component in the mixture will interact differentially between the mobile phase and adsorbent in the column. The smaller the affinity of molecule had for the stationary phase, the shorter time it spent in the column.

The adsorbent normally packed into a cylindrical column or known as packed bed chromatography. However, there are several major limitations of packed bed chromatography such as high pressure drop, flow channeling and long processing time due to limited flow rate operation. Most of this limitation can be overcome by using membrane chromatography (Ghosh, 2002). In the current study, membrane chromatography was

prepared through modification of commercial polyamide membrane by grafting methacrylic acid monomer.

1.2 Problem Statement

Packed bed chromatography used in protein separation have several limitations such as high pressure drop, increasing process time due to slow flow rate capability and flow channeling problem. Membrane chromatography is an alternative for packed bed chromatography for protein separation. Commercial and ready-made microfiltration can be transformed into membrane chromatography material by introducing specific functional group that can interact with the protein during the separation process.

In this study, the polyamide membrane was grafting with methacrylic acid to introduce carboxyl group, which can interact with the positive protein, becoming cation exchange membrane chromatography.

1.3 Research Objectives

The main objective of this study is to prepare cation exchange membrane chromatography by introducing the carboxyl group into the polyamide membrane by grafting methacrylic acid monomer.

1.4 Scope of Research

In order to fulfill the research objective, the following scopes were outlined:

i. Optimizing the attachment of carboxyl group that come from the methacrylic acid which is act as monomer by study the effect of the five different methacrylic acid concentration which is 0.1, 0.3, 0.5, 0.7 and 1.0 M during the grafting of polymer at the surface of the polyamide membrane.

ii. Study the effect reaction time to the polymer carboxyl group formation at the polyamide membrane surface with six different times which is between 15 to 120 min.

iii. Characterize the performance of the cation-exchange membrane chromatography by measuring the maximum protein binding capacity using bovine serum albumin (BSA) as the model protein.

CHAPTER 2

LITERATURE REVIEW

2.1 Membrane

Membrane can be defined as a barrier which separate two phase and restrict the transport of various chemical species in a specific manner. A membrane can be homogeneous or heterogeneous, symmetric or asymmetric in structure; it may be solid or liquid; it may be neutral, may vary between less than 100 nm to more than a centimeter of pore size. Mass transport through a membrane may be caused by convection or by diffusion or by diffusion of individual molecules, induced by electric field, or concentration, pressure or temperature gradient (Noh, 2008).

Polyamide membrane is a type of asymmetric membrane which is porous support layer from polysulfone. Polyamide membranes also comprise of a fabric and it was an ultra-thin salt rejection barrier layer. Usually polyamide membrane was used in water treatment and seawater desalination (Tarboush, 2010). The formula structure of polyamide and example of commercial polyamide membrane is showed in Figure 2.1.

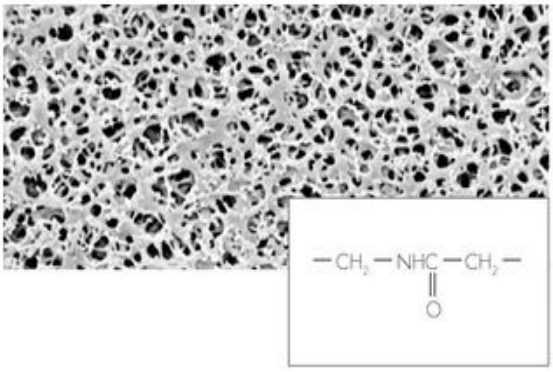

Figure 2.1: Structure of Polyamide Membrane

Polyamide membrane is a hydrophilic membrane and it also resistance to a chemical solution such as alkaline solution and organic solvent (Kasher, 2009). The recommended application of polyamide membrane is for the particle removing from water, aqueous solution and solvent. Polyamide membrane also highly recommended for the isolation of Legionella bacteria.

2.2 Ion Exchange

Ion exchange can be dividing into two types which are cation and anion exchanger. Electrostatic interaction occurs between ions in a solutions and ion in insoluble solid phase as shown in Figure 2.2.

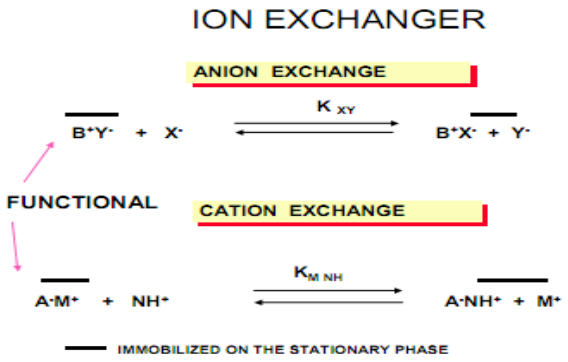

Figure 2.2: The Principle of Anion and Cation Exchanger Process

For cation exchange, the more electronegativity the cation elements in the solute, more easily the positively charge in the solid to be replaced and by following the cationic order that are $Ba^{2+} > Pb^{2+} > Sr^{2+} > Ca^{2+} > Ni^{2+} > Cd^{2+} > Cu^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+} > Ag^{+} > Cs^{+} > K^{+} > NH_4^{+} > H^{+}$. The lowest ion were chosen for the solute in order to make the exchange of ion between solid and solute exist which means the more suitable cation is hydrogen ion (H^{+}) which have the lowest electropositive.

For anion exchange, more electropositive the anion elements in solute, the negatively charged in the solid will be removed easily and the place will replace with the negatively charged of solute. To choose the lowest electronegativity of solute for anion exchange are by following the anionic order that are $SO_4^{2-} > I^{-} > NO_3^{-} > HCrO_4^{-} > Br^{-} > Cl^{-} > OH^{-}$. From the anionic order, the best ion that can be choose in order to exist the exchange between solute and solid id hydroxide ion (OH^{-}) because it has the lowest electronegativity (Geankoplis, 2003).

2.3 Protein

Proteins are macromolecules and made from amino acids which linked by covalent peptide bond (Chang & Raymond, 2003). Every protein have it unique and genetically

amino acid sequences that determining it specific shape and function such as coordinated motion, enzyme catalysis and generation and transmission (Albert et al, 2002).

Protein can be divided into three classes (Smith and Janice, 2006). First class of protein composed of long linear polypeptide chain that bundled together and form a rods or sheets. These proteins are hydrophobic and because of that, these protein roles are giving the protection to the tissues and cells and also the giving the strength.

Second class of protein is globular protein which having hydrophilic at their outer surface that makes them water soluble. Examples of this second class of protein are enzyme and transport protein. This protein is soluble in the blood and other aqueous environment in cells.

The third class of protein is a membrane protein that having role as receptors or provide channel for polar or charge molecule to passing through cell membrane.

Proteins contains positive and negative charge group depending on the amino acid sequence. Isoelectric point (pI) is the pH of the protein where the number of positive and negative charge is equal or it carries zero net charge. Table 2.1 gives the pI value of several common proteins.

Table 2.1: Isoelectric Point of Several Common Proteins

Protein	**Isoelectric pH**
Pepsin	<1.0
Ovalbumin (hen)	4.6
Serum Albumin (human)	4.9
Tropomyosin	5.1
Insulin (bovine)	5.4
Fibrinogen (human)	5.8

γ-Globulin (human)	6.6
Collagen	6.6
Myoglobin (horse)	7.0
Hemoglobin (human)	7.1
Ribonuclease A (bovine)	7.8
Histone (bovine)	10.8
Lysozyme (hen)	11.0
Salmine (salmon)	12.1

The charge of the protein depend strongly on the pH of the solution relative to it pI value as showed in Figure 2.3. If the pI of the protein below the pH of solution, the protein will has net negative charge and bind to anion-exchanger (Cutler, 2004).

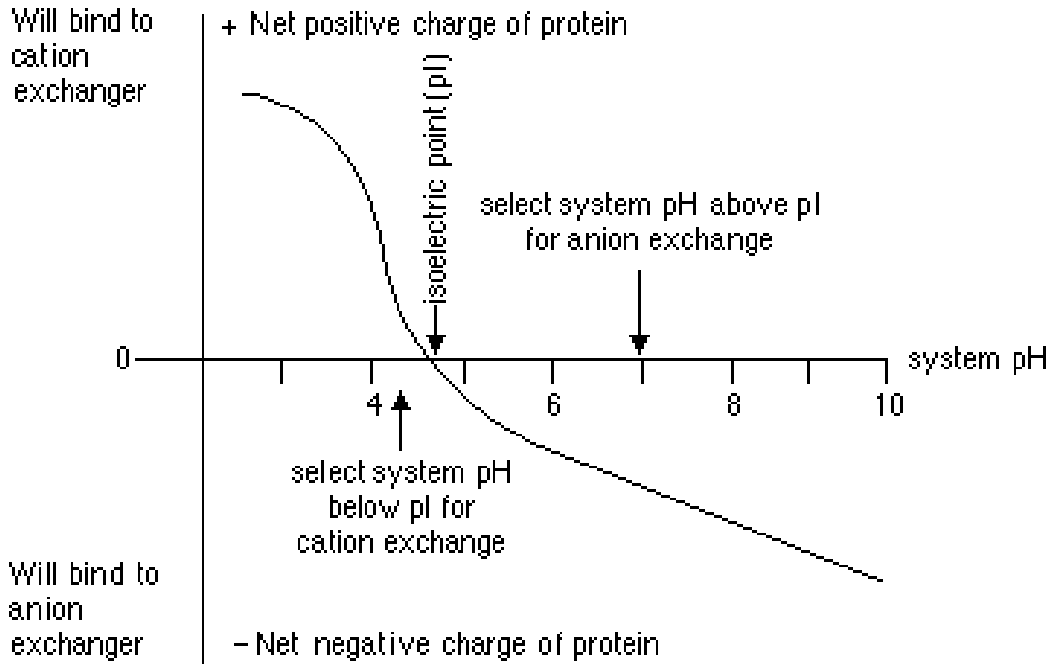

Figure 2.3: Relationship between System pH and Pi Related to the Protein Charged

2.3.1 Bovine Serum Albumin

Bovine serum albumin (BSA) is a single peptide chain that contains no carbohydrate and consisting 583 amino acids residues. The properties of BSA are showed in Table 2.2 (Haginaka, 2001).

Table 2.2: Physical Properties of Bovine Serum Albumin (Tra, 2007)

Properties	**Value**
Number of amino acid	583
Molecular Weight	69 000 Da
Isoelectric Point	4.7- 4.9
Molecular Size (Å)	40 x 40 x140

BSA is soluble in the water but can be precipitate at the high concentration of neutral salts such as ammonium sulphate. BSA has very good solution stability and because of that, BSA was used as stabilizer for other solubilized proteins such as enzyme. However, BSA will coagulate by heat starting from temperature 50°C and above and it rapidly forming hydrophobic aggregates which do not revert back to monomer upon cooling. At low temperature BSA also will coagulate but it relatively occurs in a slow rate (Burgess, 2008).

BSA is an acidic proteins group which occurs plentifully in the body fluids and tissues of mammals and in some plant seeds (Benedek, 1999). Due to the hydrophobic cleft of BSA, it can bind with water, Ca^{2+}, Na^{+}, K^{+}, fatty acids, hormones and drugs. In the biological, BSA was used to regulate the colloidal osmotic pressure blood. It also used to stabilized lipids and as blocking agent in enzyme-linked immunosorbent assay (ELISA) application.

2.4 Protein Purification

There are several techniques available for protein separation such as chromatography, membrane filtration, centrifugation, precipitation and membrane chromatography. The following section will discuss in detail about the chromatography based technique which commonly used in protein separation.

2.4.1 Chromatography

Chromatography process can be defined according to the interaction mechanism between the component and the stationary phase, or according to the type of the mobile phase used. Based on interaction mechanism, several types of chromatography such as ion exchange chromatography, hydrophobic interaction chromatography, affinity chromatography and reverse phase chromatography are available (Garret and Grisham, 2008). Figure 2.4 shows the different interaction mechanism in chromatography based separation process.

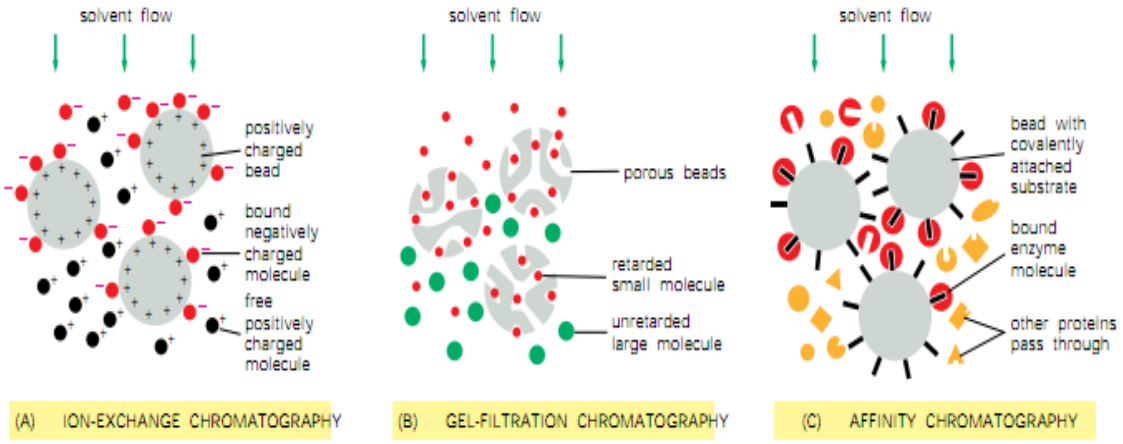

Figure 2.4: Interaction Mechanism in Chromatography Based Process

Ion-exchange chromatography media is carried certain charged either positive or negative charges. In Figure 2.4 as an example, the media had a positively charged group.

The negatively charged protein will be attracted with the media while the positively charged protein will freely flow through the chromatography media. At this condition, the positively and negatively protein can be separated.

In gel-filtration chromatography, the protein components are separated according to their size and pore size of the media. The small protein molecule can enter through the porous structure of the media beads and become delayed and travel slowly through the column. Bigger protein will excluded from entering the pore of the media and will elute early from the column.

For affinity chromatography, the ligand attached to the media had a specific interaction with the target protein molecule like a key-and-lock interaction. Therefore, only specific protein can bind to affinity chromatography and other protein will flow through the column.

2.4.2 Ion Exchange Chromatography

Two types of ion exchange chromatography available which is anion exchange chromatography and cation exchange chromatography (Stojiljkovic, 1999). Anion exchange chromatography will attract positively charged protein and cation exchange chromatography will attract negatively charged protein. The charged of the protein will depend on the isoelectric point (pI) and the pH of the operation.

Separation in ion exchange chromatography depends upon the reversible adsorption of charged solute molecules to immobilized ion exchange groups of opposite charge. Most ion exchange experiments are performed in five main stages (Harris, 2003) as shown in Figure 2.5.

In first step, an anion-exchange column was equilibrated with the suitable buffer solution. Anion-exchange was equilibrating with the buffer solution in order to increase the rate of transfer of the exchanger. The second stage is then negatively charged solute

components exchanging the ion position with the buffer solution and the salt gradient were applied. The protein component which have the lower affinity eluted first in the column by the lower concentration of the salt solution. In the fourth stage, the higher affinity of the protein component eluted after all the lower affinity of the protein component eluted. These high affinity protein components were eluted by a higher concentration of the salt solution. At the last stage, the column was equilibrated with the starting buffer and then was followed by the regeneration of the column.

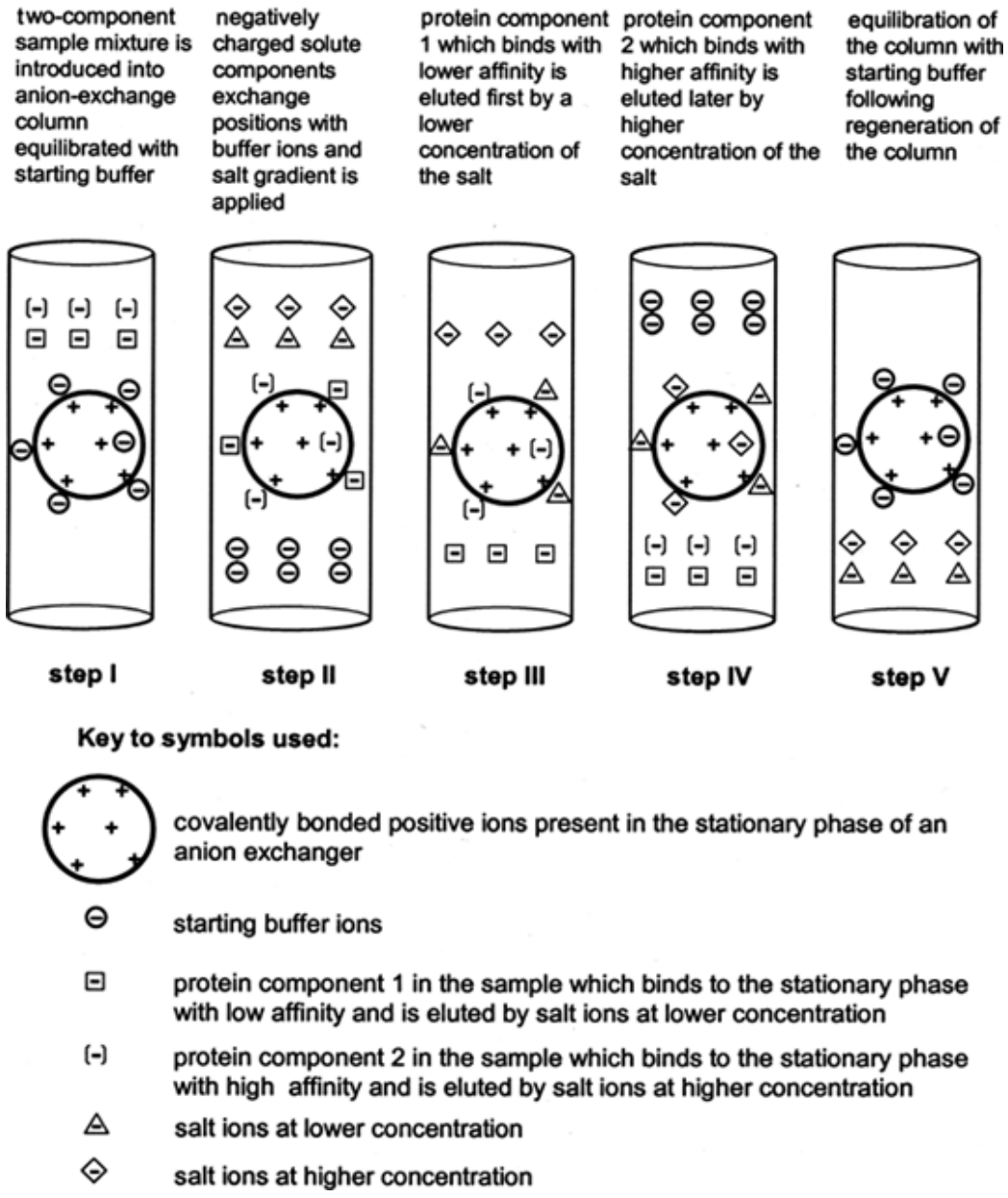

Figure 2.5: Principle of Ion Exchange Chromatography **(**Amersham Bioscience, 2008)

2.4.3 Packed Bed Chromatography

Chromatography media normally packed into cylindrical column or known as packed bed chromatography. However, there is several major limitation of packed bed configuration especially regarding to high pressure drop across the packed bed column

(Ghosh, 2002) and it tends to increase because of the combined effect of bed consolidation (Kawai et al, 2003).

Packed bed column also suffered from chanelling problem due to the flow passage through the cracked packed bed. This also will make the scale up process more complicated. The process time also increase in packed bed due to the limitation of using high flow rate to avoid excessive pressure drop. Moreover, the pore inside the packed bed was too samll and it cannot be used in the separation of the larger protein size (Zou et al, 2001).

2.4.4 Membrane Chromatography

The limitation of the packed bed chromatography can be overcome by using a membrane chromatography (Ghosh, 2002, Ghosh and Wong, 2006). The mass transfer resistance can be reduced by membrane chromatography but the diffusion transport is not totally absent. Normally, three main shapes of the membranes is used in membrane chromatography for the protein separation which are flat sheet, hollow fiber and radial flow (Cao, 2005). Membrane chromatography has an ability to purify a large biomolecules because of the open pores of the structure offers a three-dimensional structure. A channel was created from the large pore size for immediately available the active chemistry groups on the membrane surface and it will allow the high binding capacity of the large protein particles (Sellick, 2006). Other than that, the membrane chromatography can overcome the highly pressure drop in the packed bed chromatography because membrane chromatography can be operated at high flow rate and this will also reducing the process time (Ghosh, 2002)

Membrane chromatography can also be used for the larger scale of the separation process including isolation, purification and recover high purity of the protein and enzymes (Zeng and Ruckensein, 1989). Figure 2.6 shows the fluid flows inside the packed bed chromatography and membrane chromatography.

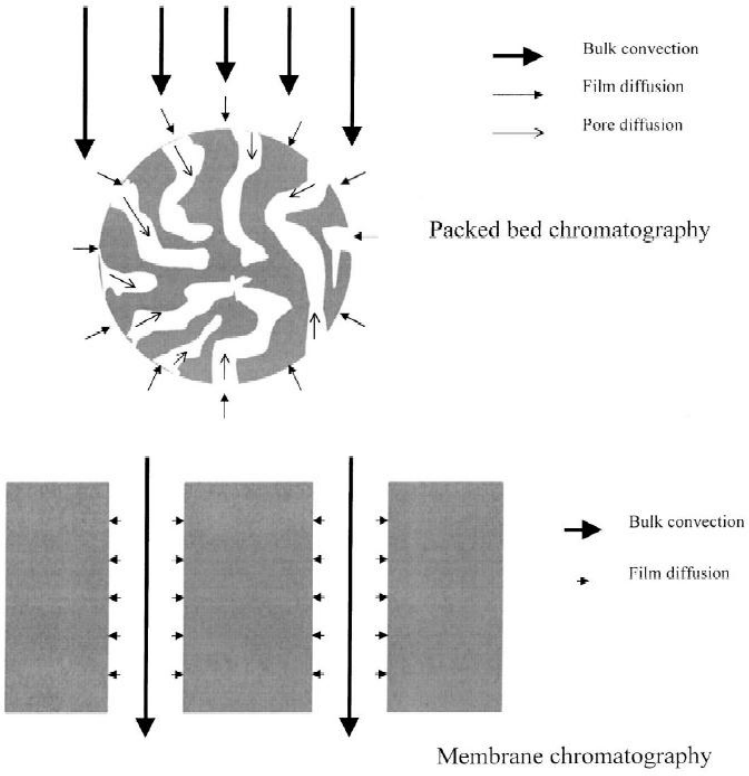

Figure 2.6: Solute Transport in Packed Bed Chromatography and Membrane Chromatography **(**Saxena et. al, 2009)

2.5 Development of Ion Exchange Membrane Chromatography

Several techniques have been used to prepare cation-exchange membrane chromatography. Some of them was summarized in Table 2.3. Hwang et al (1999) produced cation exchange membrane for electrodialysis process by sulfonation of polysulfone and polyphenylenesulfone. The membrane produced has a transport number of the cation in range 0.77 to 0.87 and ion exchange capacity of 1.9meq/(g-dry-resin).

Fang et al (2004) studied the sulfonation process and membrane preparation process during preparation of polysulfone cation-exchange membrane. In this study, they found the most favourable sulfonation condition was at 75ºC for 4 hours and the ratio of the